Toxicological Studies of the Rubber
(Hevea brasiliensis) Seed Oil

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SUMMARY

Toxicological studies were carried out on rubber Hevea brasiliensis seed oil. The study includes estimation of the cyanide content using a simple method that involves extraction of linamarase from cassava cortex to liberate the cyanide in the oil, and the detection of mycotoxins. The fresh oil contained no mycotoxins (Aflatoxin and ochratoxin) and had low cyanide concentration (0.009 - 0.013 ppm).

The implication of this study is a strong recommendation for use of this unconventional vegetable oil in animal experiments and a gradual introduction in the vegetable oil market.

INTRODUCTION

The natural occurrence of cyanide and mycotoxin in a wide range of fatty products has been reported (1) but to our knowledge few references dealing with toxins in rubber seed oil can be found in the literature. In 1986 Giesemann et al (2) reported the production of a blue fluorescing phytoalexin induced by various fungi in rubber leaves. Okoye (3) also in 1986 reported the presence of Aflatoxin B2 in stored rubber seed. Fungi contamination has been frequently reported in stored rubber seeds and defatted meal (3). Lim (4) in 1975 isolated Aspergillus niger, A. flavus, A. fumigatus, Penicillium species, rhizopus species and mucor species both outside and inside the seed as well as within the kernel.

Hevea brasiliensis para rubber has been found to be a good source of vegetable oil (5) and its physiochemical properties have been reported (6,7). The oil resembles flax oil and soyabean oil (8) and the oil has found various industrial uses (9). The oil contains hydrogen cyanide (10) which makes it unfit for human consumption. The hydrogen cyanide has been found to decrease during storage and can be completely removed during drying of the seed at 105°C and during refining (11,12).

Nevertheless we thought it worthwhile to determine the cyanide levels and the toxins present in fresh crude and refined rubber seed oil, as
refining has been found to improve the quality of vegetable oil and to remove some mycotoxin from contaminated oil (1).

Therefore this paper comprises of two distinct parts:
(a) the description of a simple method for detection of cyanide in vegetable oil and (b) an investigation of the toxins in fresh crude and refined rubber seed oil collected from Rubber Research Institute of Nigeria, Benin-City.

MATERIALS AND METHODS

Materials
Glass plates 20 x 20 cm, Quickfit applicator, desiccator, spotting template microsyringe, 10 μl, Desga developing tank, Longwave ultraviolet lamp, 366nm, Orion™ 920 ion analyser, water bath, centrifuge and ultrafiltration apparatus, Gallemkamp hot box.

All reagents used were of analytical grade including petroleum ether (40 - 60°C), benzene, toluene and formic acid.

Extraction solvent for mycotoxin detection
Methanol - water solution (6:4 v/v) containing 4% NaCl, 4% NaCl aqueous solution. Clean up solution was 20% lead acetate prepared from Pb(CH3COO)2.3H2O. Drying reagent was anhydrous sodium sulfate; Silica gel for TLC HR 60 Merck. Developing reagent used were benzene - hexane (3:1 v/v), Toluene - ethylacetate - formic acid (6:3:1 v/v/v) and ethylacetate - formic acid (99:1). Extraction solution for enzyme 0.1M phosphate buffer (pH 5.5).

Extraction of linamarase enzyme
Extraction procedure followed the method of Cooke (13). 100g of fresh cassava cortex was homogenized in 30 ml pre-chilled 0.1M phosphate buffer pH 5.5 and filtered through in sieve cloth. The resultant supernatant was centrifuged for 30 minutes 10,000xg and the supernatant concentrated through an ultra-filtration membrane (x M50). The filtrate was collected and used as crude linamarase enzyme.

Estimation of cyanide in rubber seed oil
Into a capped conical flask, 1 gm of the oil was dissolved with 30 ml of petroleum ether (40 - 60°C), and 1 ml of enzyme aliquot added. The mixture was stirred for about 2 minutes and allowed to stand for 30 minutes. The reaction was stopped by adding 1 ml of 10% NaOH. The concentration was read off in an Orion™ 920 expandable ion analyser. The procedure was the same for the blank except that the oil was omitted.

Detection of toxins:
A simple method of detection of toxins in vegetable oil as described by Litchfield and Wilcoxon (14) 1949 was used. Two clean petri-dishes were sterilized in an oven at 150°C for 1 hour and transferred to the bench.
in a pouring room until they were cooled. A sterilized knife was used to break an egg into the petri-dish. The egg yolk was removed with a sterilized spoon and few drops of the oil added to the petri-dish containing the egg albumin and the plates covered and incubated at 37°C for 24 hours.

Extraction and clean up of mycotoxins

Extraction procedure followed the method of Toussanint et al as reported by Lettotor et al (1) 1983. Into a 500ml separation funnel, 50g of oil was mixed with 100ml of hexane. Extraction was first carried out with three 10ml portions of methanol-water extraction solvent containing 4% NaCl. This was followed by extraction with two 50ml portions of aqueous 4% NaCl. After shaking for 2 minutes the aqueous and methanolic extracts were put in a separating funnel and washed twice with 50ml hexane. The aqueous-methanolic phase was transferred to a beaker and 20 ml clean up solution (20% lead acetate aqueous solution) was added and stirred well. The solution was filtered in a Buchner funnel and the precipitate washed with 50ml deionized water. The filtrate were transferred to a separating funnel and extracted with 20ml portion of chloroform. The chloroform fractions were combined, dried over anhydrous sodium sulfate, filtered and the chloroform extract evaporated to dryness using a rotary evaporator. The final volume of the extract was adjusted to 500μl with benzene. Thin layer chromatography (TLC) 20 x 20 cm glass plates coated with 0.25 mm silica gel HR 60 Merck were prepared following the direction of the manufacturer. The plates were activated by heating for 90 minutes at 105°C and allowed to cool to room temperature in a desiccator. The extracts and mycotoxin standard (Aflatoxin B₁) were spotted in TLC plates as shown in Table 1.

The plates were first developed with benzene-hexane (3:1 v/v). In this system, lipids move with the solvent front and some pigments are also eluted, while mycotoxins remain at the origin (1). After development, the plates were dried at 60°C in a Gallemkamps oven for 5 minutes and allowed to cool for a few minutes (2 minutes) in a desiccator. A second development in toluene-ethylacetate-formic acid (6:3:1) will separate mycotoxins. After drying, the plates were examined at long wavelength uv light (366nm).

Aflatoxin B₁ standard fluoresces blue and ochratoxin A standard should appear as a greenish blue spot. For confirmation of aflatoxin B₁ using plates prepared as mentioned above, it is necessary to place two 4μl on each extract and on one superimpose 2μl spot of aflatoxin B₁ (1μg/ml). Then on the 2 spots add 2μl trifluoroacetic acid and allow to react for 5 minutes. Blow warm air over the plate to remove all unreacted trifluoroacetic acid. Develop sample first on benzene-hexane to eliminate lipid and some pigments, then with ethylacetate-formic acid (99:1). This double elution leads to a clearer chromatograph. Determine whether blue fluorescent derivative of aflatoxin B₁ is present in reacted sample extract line by comparison with superimposed standard line. This will confirm identity of aflatoxin B₁ in sample extract.
RESULTS AND DISCUSSION

Mycotoxins were not detected on the fresh rubber seed oil as is evident by the absence of a blue fluorescence when viewed at long wavelength (uv) light at 366nm, and possibly supports earlier work where mycotoxins could not be detected even at 100/μl dilution (3). The hydrogen cyanide concentration of crude and refined rubber seed oil is shown in Table 2. From the table the cyanide concentration in the oil varied from 0.009 - 0.013 ppm suggesting low cyanide levels which can easily be detoxified by the biological system, and is comparable to other seed oils such as monodora myristica seed oil with cyanide level of 0.009 ppm (15).

On the qualitative estimation of toxins, the simple method could not detect the presence of toxins as is evident by the absence of green colouration in the egg albumen.

Rubber seed oil could therefore be tried in animal experiments and could equally be introduced gradually into the edible vegetable oil market.

<table>
<thead>
<tr>
<th>Spot Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>Aflatoxin B₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1μg/ml</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Unknown Samples</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude oil</td>
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<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Refined oil</td>
<td></td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
</tbody>
</table>

**TABLE 1:** Volume (μl) of Mycotoxin Standard Solution and Sample Extract on Each Plate.

**TABLE 2:** Toxicological Studies on Rubber Seed Oil

<table>
<thead>
<tr>
<th>Test</th>
<th>Crude Oil</th>
<th>Refined Oil</th>
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</thead>
<tbody>
<tr>
<td>Cyanide level</td>
<td>0.013 ppm</td>
<td>0.009 ppm</td>
</tr>
<tr>
<td>Dose effect test</td>
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<td>-ve</td>
</tr>
<tr>
<td>Aflatoxin B₁</td>
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<td>Not detected</td>
</tr>
<tr>
<td>Ochratoxin</td>
<td>Not Detected</td>
<td>Not Detected</td>
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The results are average of triplicate analysis.
ACKNOWLEDGEMENT

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REFERENCES


